UNITED STATES ENVIRONMENTAL PROTECTION AGENCY

OFFICE OF CHEMICAL SAFETY
AND POLLUTION PREVENTION

December 16, 2020

MEMORANDUM

SUBJECT: Memorialization of EPA's process and rationale for reaching its "No Effect" finding under the Endangered Species Act in its risk assessment supporting the Experiment Use Permit (EUP) issued under Section 5 of the Federal Insecticide, Fungicide, and Rodenticide Act for the release of OX5034 *Aedes aegypti* mosquitoes (EPA File Symbol: 93167-EUP-E) containing Tetracycline Trans-Activator Variant (tTAV-OX5034) protein and the genetic material (from vector pOX5034) necessary to produce the protein in OX5034 *Aedes aegypti*.

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This memorandum is a memorialization of EPA's process and rationale for reaching its "No Effect" finding in its risk assessment supporting the Experiment Use Permit (EUP) issued on April 30, 2020, under Section 5 of the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) for the release of OX5034 *Aedes aegypti* mosquitoes containing Tetracycline Trans-Activator Variant (tTAV-OX5034) protein and the genetic material (from vector pOX5034) necessary to produce the protein *in vivo* (hereinafter referred to more simply as OX5034 *Ae. aegypti* mosquitoes). This memorialization is based wholly on materials found in the decisional record for this EUP.

The EUP, which is authorized only in the states of Florida and Texas, will evaluate efficacy of the release of OX5034 *Ae. aegypti* mosquitoes against wild *Aedes aegypti* mosquitoes (hereinafter referred to as *Ae. aegypti* mosquitoes) within Monroe County, Florida and Harris County, Texas. Shipment and/or use under this permit are subject to the provisions of 40 CFR Part 172.

As part of EPA's risk assessment[1], per Section 7 of the Endangered Species Act (ESA), EPA considered whether the issuance of the EUP would jeopardize the continued existence of any threatened or endangered species or result in the destruction or adverse modification of the critical habitat of such species.

After careful consideration of potential direct or indirect interactions that OX5034 *Ae. aegypti* mosquitoes may have with nontarget organisms (i.e., organisms that are not *Ae. aegypti*), EPA concluded that no adverse effects are anticipated for any nontarget organisms as a result of the experimental permit to release OX5034 *Ae. aegypti* mosquitoes based on the screening level assessment that showed no risks of concern at the taxa level. This finding means that there are no discernible effects to nontarget organisms reasonably expected to occur within the action area. As no adverse effects are anticipated for any nontarget organism (i.e., no discernible effects to nontarget organisms are reasonably expected to occur within the action area),[2] which necessarily includes any threatened or endangered species (listed species), EPA therefore reached a "No Effect" determination for direct and indirect effects to listed species, and their critical habitats.

In this memorandum, EPA provides brief summaries of key considerations used in the Agency's determination that no adverse effects are anticipated for nontarget organisms as a result of the experimental permit to release OX5034 *Ae. aegypti* male mosquitoes and in the Agency's "No Effect" determination for direct and indirect effects to listed species, and their critical habitats.

Background information

The pesticidal effect of OX5034 is species-specific and results in emergence of all-male progeny in the absence of a dietary antidote in the larval diet. The pesticidal effect of OX5034 is species-specific as it only affects the reproductive success of *Ae. aegypti* mosquitoes through mating between OX5034 *Ae. aegypti* males and *Ae. aegypti* females that are already present in the release area. During the EUP, only OX5034 *Ae. aegypti* male mosquitoes will be released into the environment. Only female offspring from OX5034 matings fail to mature to adulthood due to the genetic construct, while male offspring containing the OX5034 genetic construct survive to further pass on the OX5034 female-lethal trait. Unlike female mosquitoes, male mosquitoes do not bite humans or animals. With continued field releases of OX5034 *Ae. aegypti* males, the *Ae. aegypti* population in the treatment area is expected to progressively decline due to the reduced number of females remaining in the wild population.

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[1] *See* EPA, Human Health and Environmental Risk Assessment for the New Product OX5034 Containing the Tetracycline-Repressible Transactivator Protein Variant (tTAV-OX5034; New Ingredient Protein, a DsRed2 Protein Variant (DsRed2-OX5034; New Inert Ingredient), and the Genetic Material (Vector pOX5034) Necessary for Their Production in OX5034 *Aedes aegypti*; Data and Information Were Provided in Support of a FIFRA Section 5 Application (hereinafter Human Health and Environmental Risk Assessment), Pg. 43-49, *available at* https://beta.regulations.gov/document/EPA-HQ-OPP-2019-0274-0359.

[2] The conclusion of the Risk Assessment that no adverse effects are anticipated for any nontarget organisms is intended to convey that there are no discernible effects to nontarget organisms reasonably expected to occur within the action area. That is the meaning of the term "no adverse effects" throughout this document.

Although the EUP is granted for only limited acreage and a limited release design[3], the release could result in population level decline of *Ae. aegypti* in the testing area. Therefore, EPA considered not only the possibility of traditional adverse effects to nontarget organisms (i.e., direct effects from oral consumption), but also the possibility of indirect effects on ecosystem processes from reduced *Ae. aegypti* populations.

As part of the problem formulation of EPA's risk assessment, EPA postulated potential routes of exposure and potential hazards that warranted further consideration, as stated in the risk assessment: "[p]ossible adverse effects to non-target organisms from OX5034 releases are two-pronged: direct effects from oral consumption of OX5034 mosquitoes and indirect effects on ecosystem process from reduced *Ae. aegypti* population." EPA then evaluated these postulations in the risk assessment and determined that no adverse effects are anticipated for any nontarget organisms at the taxa level, which necessarily includes any listed species, and therefore reached a "No Effect" determination for listed species and their critical habitats.

During the EUP evaluation, EPA considered possible routes of exposure to OX5034 *Ae. aegypti* male mosquitoes, the likelihood of a hazard from the consumption of OX5034 *Ae. aegypti* male mosquitoes, and the likelihood of a hazard from the possible reduction in the wild *Ae. aegypti* population leading to a possible reduction in a nontarget organism's food source. EPA then evaluated risk by examining the possible hazards and possible routes of exposure in conjunction (i.e., Risk = Hazard x Exposure). In events where exposure may be possible, but no hazard is identified, risk is concluded to be negligible.

A summary of EPA's evaluation of possible routes of exposure

Two postulated routes of exposure are dermal exposure and oral exposure. Given that DsRed2-OX5034 and tTAV-OX5034 are expressed in OX5034 tissues within the confines of its chitinaceous exoskeleton, both proteins are unavailable to nontarget organisms dermally should OX5034 males merely land on the surface of a nontarget organism[4]. Female mosquitoes (non-species specific) take blood meals from humans and other animals, but because biting females will not be released under the approved EUP and male mosquitoes do not bite animals, nontarget organisms including listed species will not serve as bloodmeals for mosquitoes carrying tTAV-OX5034 and DsRed2-OX5034 proteins.

As described in EPA's risk assessment[5], OX5034 female lethality is attributed to the overexpression of the tTAV-OX5034 protein in immature females, a process that is thought to interfere with the transcriptional machinery of the insect and consequently normal cellular function. As a result, females carrying the OX5034 trait survive only until the early larval stages unless tetracycline, which acts as a dietary antidote, is present at high enough levels. EPA confirmed via results from studies using mosquitoes from laboratory colonies and from field collections, that the OX5034 phenotype is 100% penetrant and that all females containing a copy of the OX5034 trait die prior to adulthood when reared in

[3] *See* EPA, Review of Updated Section G for an Experimental Use Permit 93167-EUP-E to Test OX5034 *Aedes aegypti* Mosquitoes Decision #549240; Submission #1047971, *available at* https://beta.regulations.gov/document/EPA-HQ-OPP-2019-0274-0352.
[4] *See* EPA, Human Health and Environmental Risk Assessment, Pg. 47, *available at* https://beta.regulations.gov/document/EPA-HQ-OPP-2019-0274-0359.
[5] *See* EPA, Human Health and Environmental Risk Assessment, Pg. 12, *available at* https://beta.regulations.gov/document/EPA-HQ-OPP-2019-0274-0359.

the absence of a tetracycline analogue[6]. Because the presence of tetracycline(s) in the environment may affect survivability of female OX5034 mosquitoes, the likelihood that OX5034 mosquitoes would encounter tetracycline sources at levels high enough for rescue from the lethal phenotype was evaluated[7]. Several lines of evidence including a survey of environmental levels of tetracycline, tetracycline dose-response testing of OX5034 females, and oviposition behavior of *Ae. aegypti*, indicate that the risk of hemizygous OX5034 female mosquitoes emerging in the environment due to high levels of tetracycline is low[8]. A term of the EUP restricts releases from occurring within 500 meters of potential tetracycline sources (i.e., sewage treatment facilities and any farms producing citrus crops) [9], resulting in the determination that the exposure to female mosquitoes is negligible[10] and the exclusion of female biting as a dermal exposure pathway to the tTAV-OX5034 and DsRed2-OX5034 proteins.

Regarding possible oral exposure, insect-eating animals, by definition, eat insects, and as mosquitoes are an insect it is possible for mosquitoes to be consumed by insect-eating animals. Given that it is possible that insect-eating animals might consume OX5034 *Ae. aegypti* male mosquitoes, EPA performed a comprehensive evaluation of the likely routes of exposure to OX5034 *Ae. aegypti* male mosquitoes for nontarget organisms, which includes listed species, as summarized below.

Ae. aegypti is a major disease vector for humans as it is known to vector diseases such as yellow fever, Zika, chikungunya, and dengue[11,12]. The same dietary and habitat preferences that make *Ae. aegypti* females a deadly vector to humans are what limit the exposure of nontarget organisms to OX5034 *Ae. aegypti* male mosquitoes. Female *Ae. aegypti* preferentially feed on humans[13] and therefore prefer that their breeding locations be near human dwellings in order to be in close proximity to their preferred food source[14]. As such, *Ae. aegypti* usually uses man-made containers such as gutters, water containers, cans, and tires as breeding sites, and adults typically rest on walls and in shaded areas within and around human dwellings[15,16,17]. It is relevant to note that the Hribar *et al.* 2001 and Hribar *et al.* 2004 studies referenced

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[6] EPA, The self-limiting phenotype, penetrance, longevity and egg clutch size of *Aedes aegypti*, OX5034; Evaluation of field penetrance of OX5034 in open release field trials in Indaiatuba, Sao Paolo State, Brazil; Supplemental information in support of the study, evaluation of field penetrance of OX5034 in open release field trials in Indaiatuba, Sao Paolo State, Brazil. MRIDs: 50889417, 50889423, 50889428.
[7] *See* EPA, Human Health and Environmental Risk Assessment, Pg. 31-34, *available at* https://beta.regulations.gov/document/EPA-HQ-OPP-2019-0274-0359.
[8] EPA, Dose Response of Hemizygous Aedes aegypti OX5034 to Tetracyclines and Effects of Environmental Exposure to Tetracyclines. MRID: 50889415.
[9] *See* EPA, Experimental Use Permit Issued for 93167-EUP-2 to Allow for Releases of OX5034 *Aedes aegypti* in Florida and Texas, Pg. 3., *available at* https://beta.regulations.gov/document/EPA-HQ-OPP-2019-0274-0353.
[10] *See* EPA, Human Health and Environmental Risk Assessment, Pg. 50, *available at* https://beta.regulations.gov/document/EPA-HQ-OPP-2019-0274-0359.
[11] Nelson, M. J. 1986. Aedes aegypti: Biology and Ecology, Pg. 2-3, Washington, D.C.
[12] Christophers R. 1960. Aedes aegypti (L.) The Yellow Fever Mosquito: Its Life History, Bionomics and Structure. Pg. 77-83, Cambridge University Press.
[13] Harrington, L. C., J. D. Edman, and T. W. Scott. 2001. Why do female Aedes aegypti (Diptera: Culicidae) feed preferentially and frequently on human blood? Journal of Medical Entomology 38:411-422.
[14] Nelson, M. J. 1986. Aedes aegypti: Biology and Ecology, Pg. 4, Washington, D.C.
[15] TunLin, W., B. H. Kay, and A. Barnes. 1995. Understanding productivity, a key to *Aedes aegypti* surveillance. American Journal of Tropical Medicine and Hygiene 53:595-601.
[16] Hribar, L. J., J. M. Smith, J. J. Vlach, and T. N. Verna. 2001. Survey of container-breeding mosquitoes from the Florida Keys, Monroe County, Florida. Journal of the American Mosquito Control Association 17:245-248.
[17] Christophers R. 1960. Aedes aegypti (L.) The Yellow Fever Mosquito: Its Life History, Bionomics and Structure. Cambridge University Press. Pg. 57-59.

in EPA's risk assessment[18] are mosquito habitat surveys specific to one of the EUP locations (Monroe County, FL). Therefore, findings such as "[o]n Big Pine Key and Vaca Key, mosquito larvae were most often collected from tires, whereas on Key West most collections were made from flowerpots, planters, and trivets"[19] described in Hribar *et al.* 2004 provide additional certainty that habitat preferences of *Ae. aegypti* described in the general scientific literature also hold true at the EUP locations. EPA therefore concluded that the use of these man-made containers as larval habitat and breeding sites greatly reduces the likelihood of nontarget organisms, which includes listed species, encountering OX5034 *Ae. aegypti* larvae.

EPA stated in its risk assessment: "Originating in sub-Saharan Africa, *Ae. aegypti* is believed to have been introduced to the Americas in the 17th century. While *Ae. aegypti* historically bred in tree holes and other phytotelmata, it is now well adapted to humans, flourishes in urban areas, and can breed in a number of artificial containers."[20] It is possible for *Ae. aegypti* in the United States to also use tree holes or rock holes as breeding sites, but due to *Ae. aegypti's* high affinity for humans in the Americas[21], *Ae. aegypti* is rarely found more than 100 meters from human dwellings[22]. This proximity to human dwellings further reduces the likelihood of OX5034 *Ae. aegypti* larvae encountering nontarget organisms even in instances where the OX5034 larvae are found in more natural habitats such as tree holes or rock pools.

The anthropophilic nature of *Ae. aegypti* mosquitoes also reduces the likelihood of nontarget organisms encountering OX5034 *Ae. aegypti* adult males. This is because *Ae. aegypti* adults are typically found near or even inside human dwellings, thus limiting their availability to predators[23,24]. As previously discussed, *Ae. aegypti* are adapted to domestic and urban environments that allow females easy and unlimited access to blood meals, such as those around human habitations. Although only females take blood meals, *Ae. aegypti* males also frequent human habitations in order to maintain proximity to females for mating[25]. Due to the anthropophilic nature of the target pest, OX5034 *Ae. aegypti* releases will occur in residential sites[26]. As *Ae. aegypti* dispersal is generally limited to around 200 meters based on worldwide release recapture studies[27], released OX5034 *Ae. aegypti* will not travel far from the release site.

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[18] *See* EPA, Human Health and Environmental Risk Assessment, Pg. 32-33, *available at* https://beta.regulations.gov/document/EPA-HQ-OPP-2019-0274-0359.

[19] Hribar, L. J., J. J. Vlach, D. J. DeMay, S. S. James, J. S. Fahey, and E. M. Fussell. 2004. Mosquito larvae (Culicidae) and other Diptera associated with containers, storm drains, and sewage treatment plants in the Florida Keys, Monroe County, Florida. Florida Entomologist 87:199-203. Pg. 201.

[20] *See* EPA, Human Health and Environmental Risk Assessment, Pg. 44 (footnotes omitted), *available at* https://beta.regulations.gov/document/EPA-HQ-OPP-2019-0274-0359.

[21] Powell, J. R., and W. J. Tabachnick. 2013. History of domestication and spread of *Aedes aegypti* - A Review. Memorias Do Instituto Oswaldo Cruz 108:11-17.

[22] Nelson, M. J. 1986. Aedes aegypti: Biology and Ecology, Pg. 4, Washington, D.C.

[23] Christophers R. 1960. Aedes aegypti (L.) The Yellow Fever Mosquito: Its Life History, Bionomics and Structure. Cambridge University Press. Pg. 59.

[24] EPA, Tetracycline-Repressible Transactivator Protein Variant (tTAV-OX5034) and Related Genetic Material from OX5034 *Aedes aegypti*: Request for Wavier from the Wild Mammal Toxicity Testing. Volume 23, EUP Submission; MRID 50889422, July 16, 2019. Pg. 4.

[25] Nelson, M. J. 1986. Aedes aegypti: Biology and Ecology, Pg. 9, Washington, D.C.

[26] *See* EPA, Review of Updated Section G for an Experimental Use Permit 93167-EUP-E to Test OX5034 *Aedes aegypti* Mosquitoes Decision #549240; Submission #1047971, *available at* https://beta.regulations.gov/document/EPA-HQ-OPP-2019-0274-0352.

[27] OECD. 2018. Safety Assessment of Transgenic Organisms in the Environment, Volume 8. Pg. 97.

Therefore, due to species-specific behavioral traits of *Ae. aegypti* resulting in its preferential habitat being largely limited to areas surrounding human dwellings and its preferential breeding sites being largely composed of man-made containers, the potential of exposure of nontarget organisms to OX5034 *Ae. aegypti* males is limited. Therefore, due to the OX5034 *Ae. aegypti* releases occurring in residential sites and to biological traits of *Ae. aegypti* (e.g., anthropophilic, limited dispersal), it is reasonable to find that exposure to OX5034 *Ae. aegypti* mosquitoes by listed species is expected to be limited.

A summary of EPA's evaluation of the likelihood of hazard through direct consumption

As described in EPA's risk assessment[28], the pesticidal effect of OX5034 is species-specific as it only affects the reproductive success of *Ae. aegypti* through mating between OX5034 *Ae. aegypti* males and *Ae. aegypti* females that are already present in the release area. OX5034 *Ae. aegypti* males will be released into the environment; quality assurance protocols will ensure no female mosquitoes are released. Only female offspring from OX5034 matings fail to mature to adulthood due to the genetic construct, while male offspring from OX5034 matings survive to further pass on the OX5034 female-lethal trait. OX5034 female lethality is intracellular and is attributed to the overexpression of the tTAV-OX5034 protein in immature females, a process that is thought to interfere with the transcriptional machinery of the insect and consequently normal cellular function. In addition to the tTAV-OX5034 protein, EPA also evaluated the DsRed2-OX5034 protein, which is a variant of the DsRed fluorescent protein form *Discosoma* spp., that allows for the visual identification of OX5034 hemizygous larvae collected from the field.

EPA evaluated whether there was any risk to nontarget organisms, which includes listed species, from the consumption of the OX5034 *Ae. aegypti* male mosquito and EPA found that no adverse effects from consumption are expected. It is important to again note that nontarget exposure to OX5034 *Ae. aegypti* male mosquitoes is expected to be limited, but in order to comprehensively evaluate potential risk to nontarget organisms, including listed species, EPA evaluated the effects of nontarget consumption of OX5034 *Ae. aegypti* male mosquitoes.

No direct adverse effects[29] due to consumption of OX5034 males by nontarget organisms are expected based on bioinformatics analyses and acute oral toxicity studies. Based upon bioinformatic analysis, neither the DsRed2-OX5034 or tTAV-OX5034 proteins share significant sequence similarity with known toxins[30,31]. Both proteins are predicted to be susceptible to several proteases found in the human digestive system (i.e., pepsin, trypsin, chymotrypsin) based upon bioinformatics analysis[32,33], and thus the proteins are expected to be broken down following ingestion.

In addition to these bioinformatics analyses, EPA also evaluated toxicity studies which indicated that fish and freshwater invertebrates that ingest OX5034 *Ae. aegypti* male mosquitoes are not adversely

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[28] *See* EPA, Human Health and Environmental Risk Assessment, Pg. 12, *available at* https://beta.regulations.gov/document/EPA-HQ-OPP-2019-0274-0359.

[29] No indirect adverse effects are expected either, as discussed in this memo in the section entitled, "A summary of EPA's evaluation of the likelihood of hazard through the potential for reduced food source."

[30] Oxitec Ltd., MRID 50889420. 2019. Bioinformatics analysis for risks of allergenicity and toxicity of proteins encoded by the two genes introduced into genetically engineered mosquitoes (*Aedes aegypti*) strain OX5034.

[31] EPA, Bioinformatics analysis for risks of allergenicity and toxicity of proteins encoded by the two genes introduced into genetically engineered mosquitoes (Aedes aegypti) strain OX5034. Volume 21. Pg. 9.

[32] Oxitec Ltd., MRID 50889420. 2019. Bioinformatics analysis for risks of allergenicity and toxicity of proteins encoded by the two genes introduced into genetically engineered mosquitoes (*Aedes aegypti*) strain OX5034.

[33] EPA, Bioinformatics analysis for risks of allergenicity and toxicity of proteins encoded by the two genes introduced into genetically engineered mosquitoes (Aedes aegypti) strain OX5034. Volume 21. Pg. 9.

affected[34,35, 36, 37]. A submitted study tested the potential toxicity of OX5034 *Ae. aegypti* male mosquitoes fed to guppies[38,39] to evaluate the direct impact of consumption of OX5034 mosquitoes on nontarget aquatic vertebrate organisms. The study found no acute or sublethal adverse effects to the test organisms over the 14-day test period, indicating that the likelihood for adverse effects to nontarget aquatic vertebrates from consumption of OX5034 *Ae. aegypti* mosquitoes is low.

To evaluate the direct impact on nontarget aquatic invertebrate organisms through oral consumption of OX5034 *Ae. aegypti*, a submitted study tested the potential toxicity of OX5034 *Ae. aegypti* male mosquitoes to an aquatic invertebrate. A feeding study examined the American signal crayfish[40,41] and found no acute or sublethal adverse effects to the test organisms when fed OX5034 mosquitoes over a 96-hour test period. As crayfish are larger in mass than juvenile aquatic insects and may therefore be less sensitive to low level toxins, EPA recommended an aquatic insect larval study be performed prior to a Section 3 registration for additional certainty regarding transferability of the study conclusions to juvenile aquatic insects. Although the use of aquatic insect larva rather than crayfish as a test organism for an aquatic invertebrate study was recommended, it should be noted that in the evaluation of the waiver for nontarget insect testing, which was deemed acceptable, EPA stated that "concerns regarding oral consumption of OX5034 mosquitoes by insect species is not considered as a significant risk due to a lack of plausible toxicity to these species via uptake during normal digestive processes."[42]

In summary, EPA concluded that no adverse effects to nontarget organisms at the taxa level, which necessarily includes listed species, are expected from the consumption of OX5034 *Ae. aegypti* male mosquitoes based on 1) bioinformatics analyses demonstrating lack of similarity between DsRed2-OX5034 or tTAV-OX5034 and known toxins, 2) bioinformatics analyses demonstrating susceptibility of DsRed2-OX5034 or tTAV-OX5034 to gastric proteases, 3) toxicity study indicating no adverse effects to fish upon OX5034 *Ae. aegypti* male mosquito consumption, and 4) toxicity study indicating no adverse effects to an aquatic invertebrate upon OX5034 *Ae. aegypti* male mosquito consumption. Therefore, as EPA concluded that no adverse effects were expected to nontarget organisms at the taxa level, a "No Effect" determination (due to consumption) was made for listed species.

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[34] Oxitec Ltd., MRID 50889407. 2019. Supplemental Information in Support of the Study, *Aedes aegypti* strain OX5034 larvae (batch RD021018): 96 Hour Feeding Study with the American (Signal) Crayfish (Envigo Study No. VH34HP; EPA MRID 50698707).

[35] Oxitec Ltd., MRID 50889408. 2019. Supplemental Information in Support of the Study, A laboratory toxicity study to determine the effects of *Aedes aegypti* strain OX5034 towards *Poecilia reticulata* (Actinopterygii: Poeciliidae) under semi-static conditions (Syntech Study No 232SRRES18C01).

[36] Oxitec Ltd., MRID 50698708. 2019. A laboratory toxicity study to determine the effects of *Aedes aegypti* strain OX5034 towards Poecilia reticulata (Actinopterygii: Poeciliidae) under semi-static conditions.

[37] Oxitec Ltd., MRID 50698707. 2019. *Aedes aegypti* strain OX5034 larvae (batch RD021018): 96 Hour Feeding Study with the American (Signal) Crayfish.

[38] Oxitec Ltd., MRID 50889408. 2019. Supplemental Information in Support of the Study, A laboratory toxicity study to determine the effects of *Aedes aegypti* strain OX5034 towards *Poecilia reticulata* (Actinopterygii: Poeciliidae) under semi-static conditions (Syntech Study No 232SRRES18C01).

[39] Oxitec Ltd., MRID 50698708. 2019. A laboratory toxicity study to determine the effects of *Aedes aegypti* strain OX5034 towards Poecilia reticulata (Actinopterygii: Poeciliidae) under semi-static conditions.

[40] Oxitec Ltd., MRID 50889407. 2019. Supplemental Information in Support of the Study, *Aedes aegypti* strain OX5034 larvae (batch RD021018): 96 Hour Feeding Study with the American (Signal) Crayfish (Envigo Study No. VH34HP; EPA MRID 50698707).

[41] Oxitec Ltd., MRID 50698707. 2019. *Aedes aegypti* strain OX5034 larvae (batch RD021018): 96 Hour Feeding Study with the American (Signal) Crayfish.

[42] EPA, Tetracycline-Repressible Transactivator Protein Variant (tTAV-OX5034) and Related Genetic Material from OX5034 Aedes aegypti: Request for Waiver from Nontarget Insect Testing MRID: 50889413. Pg. 3.

A summary of EPA's evaluation of the likelihood of hazard through the potential for reduced food source

Based on a review of the scientific literature, no published papers have been located that identify any predator species that is dependent on *Ae. aegypti* as a crucial component of its diet[43],[44]. There are over 3,000 species of mosquitoes worldwide with approximately 176 species of mosquitoes in the United States, and *Ae. aegypti* is but one. For example, *Ae. aegypti* make up less than 2% of the mosquito species caught in traps in Monroe County, Florida[45], making it unlikely that *Ae. aegypti* would play a critical role in the diet of any predators, including listed species. Nonetheless, in order to comprehensively evaluate the potential risk to nontarget organisms, including listed species, from the release of OX5034 *Ae. aegypti* male mosquitoes, EPA evaluated the potential for ecosystem level effects due to the possibility of population level reduction of the target pest. EPA provided a number of examples in its risk assessment of instances in which predators may consume mosquitoes (non-species specific) as some portion of their diet, but do not significantly rely on mosquitoes, and specifically do not rely on the *Ae. aegypti* mosquito, as a major food source. Additional examples can be found in MRID 50889414 and the Data Evaluation Record[46] reviewing the MRID[47]. Organisms that consume, or are capable of consuming, mosquitoes (non-species specific) are typically considered to be dietary generalists largely due to an individual mosquito containing little caloric energy[48], thereby making a dietary strategy that specializes on mosquitoes to be energetically costly. An example of this is a report of bat diet preferences in Florida that indicates that although the southeastern brown bats in Florida do ingest mosquitoes, they display a strong preference for beetles and moths in their diet[49], which tend to be larger prey items and thus more energetically efficient targets.

It is also pertinent to note *Ae. aegypti* is an invasive species in the United States. Given its relatively recent arrival[50], *Ae. aegypti* is therefore unlikely to represent a keystone species or to have co-evolved any significant relationships with nontarget organisms in the United States. The expected lack of significant relationships further supports the expectation that nontarget organisms do not specifically rely on *Ae. aegypti* for food.

Additionally, as *Ae. aegypti* is a major pest species with known impacts on human health by vectoring disease, it is continually suppressed by other control methods such as the use of chemical and microbial

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[43] Oxitec Ltd., MRID 50889414. 2019. Analysis of no effect to threatened or endangered species or critical habitat. Pg. 17.

[44] US Food and Drug Administration., MRID 50443513. 2016. *Environmental Assessment for Investigational Use of Aedes aegypti OX513A In support of a proposed field trial of genetically engineered (GE) male Ae. aegypti mosquitoes of the line OX513A in Key Haven, Monroe County, Florida.* Pg. 82-91.

[45] Oxitec Ltd., MRID 50889414. 2019. Analysis of no effect to threatened or endangered species or critical habitat. Pgs. 12-13.

[46] EPA, Tetracycline-Repressible Transactivator Protein Variant (tTAV-OX5034) and Related Genetic Material from OX5034 *Aedes aegypti*: Analysis of No Effect to Threatened or Endangered Species or Critical Habitat. Volume 15, EUP Submission; MRID 50889414, July 16, 2019.

[47] Additional examples and rationale can be found in MRIDs 50889409, 50889410, 50889411, 50889412, 50889413, 50889422 and their associated Data Evaluation Records.

[48] Wetzler, G. C., and J. G. Boyles. 2018. The energetics of mosquito feeding by insectivorous bats. Canadian Journal of Zoology 96:373-377. Pg. 373-374.

[49] Zinn, T. L., and S. R. Humphrey. 1981. Seasonal Food Resources and Prey Selection of the Southeastern Brown Bat *Myotis austroriparius* in Florida USA. Florida Scientist 44:81-90. Pg. 87.

[50] Powell, J. R., and W. J. Tabachnick. 2013. History of domestication and spread of *Aedes aegypti* - A Review. Memorias Do Instituto Oswaldo Cruz 108:11-17. Pg. 12.

insecticides as well as breeding site source reduction[51], which further reduces the likelihood that a predator would be dependent on *Ae. aegypti* as a food source.

Moreover, the species-specific behaviors of *Ae. aegypti* outlined as factors limiting exposure to nontarget organisms also limit the likelihood that predators would be reliant on this species. To this point, aquatic predator species tend to be rare or absent from man-made containers[52,53]. Because *Ae. aegypti* usually uses man-made containers such as gutters, water containers, cans, and tires as breeding sites, there appears to be no specific predator that preys exclusively on *Ae. aegypti* in the aquatic stage, but, rather, predators that are generally opportunistic and feed on larvae or adults when they encounter them[54]. This rationale also applies to adult *Ae. aegypti*, as they are typically found near or even inside human dwellings, thus providing some protection from predators[55,56]. Moreover, as required by the anthropophilic nature of the target pest, OX5034 *Ae. aegypti* releases will occur in residential sites [57]. As *Ae. aegypti* dispersal is generally limited to around 200 meters based on worldwide release recapture studies[58], released OX5034 *Ae. aegypti* will not travel far from the release site, therefore restricting access to predators.

In summary, EPA concluded that no adverse effects to nontarget organisms at the taxa level, which necessarily includes listed species, are expected should OX5034 *Ae. aegypti* male mosquitoes successfully reduce the *Ae. aegypti* population in the EUP locations based on 1) literature reviews that indicate that no species are reliant on *Ae. aegypti* mosquitoes as a food source, 2) the generalist nature of predators that consume mosquitoes, 3) species-specific behavioral traits of *Ae. aegypti* that limit the potential for interaction with nontarget organisms, 4) the invasive species status of *Ae. aegypti* which reduces the likelihood that any significant co-evolutionary relationships exist with nontarget organisms in the United States, and 5) *Ae. aegypti* is commonly targeted for pest reduction through mosquito control measures which further limits the likelihood that a nontarget organism would be reliant upon this species for food. Therefore, as EPA concluded that no adverse effects were expected to nontarget organisms at the taxa level, a "No Effect" determination (due to population reduction) was made for listed species.

A summary of EPA's determination

As described in this memorandum, EPA considered possible routes of exposure to OX5034 *Ae. aegypti* male mosquitoes, the likelihood of a hazard from the consumption of OX5034 *Ae. aegypti* male mosquitoes, and the likelihood of a hazard from the possible reduction in the wild *Ae. aegypti* population leading to a possible reduction in a nontarget organism's food source. EPA then evaluated risk by

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[51] Nelson, M. J. 1986. Aedes aegypti: Biology and Ecology, Washington, D.C. Pgs. 30-34.
[52] Kesavaraju B., Damal K., Juliano SA. 2008. Do natural container habitats impede invader dominance? Predator-mediated coexistence of invasive and native container-dwelling mosquitoes. Oecologia 155:631–9. DOI: 10.1007/s00442-007-0935-4. Pg. 6.
[53] Juliano SA. 2009. Species interactions among larval mosquitoes: Context dependence across habitat gradients. Ann Review Entomology 54:37–56. DOI: 10.1146/annurev.ento.54.110807.090611. Pg. 7.
[54] Christophers R. 1960. Aedes aegypti (L.) The Yellow Fever Mosquito: Its Life History, Bionomics and Structure. Cambridge University Press. Pg. 59.
[55] Nelson, M. J. 1986. Aedes aegypti: Biology and Ecology, Washington, D.C. Pg. 4, 12.
[56] EPA, Tetracycline-Repressible Transactivator Protein Variant (tTAV-OX5034) and Related Genetic Material from OX5034 *Aedes aegypti*: Request for Wavier from the Wild Mammal Toxicity Testing. Volume 23, EUP Submission; MRID 50889422, July 16, 2019. Pg. 4.
[57] *See* EPA, Review of Updated Section G for an Experimental Use Permit 93167-EUP-E to Test OX5034 *Aedes aegypti* Mosquitoes Decision #549240; Submission #1047971, *available at* https://beta.regulations.gov/document/EPA-HQ-OPP-2019-0274-0352.
[58] OECD. 2018. Safety Assessment of Transgenic Organisms in the Environment, Volume 8. Pg. 97.

examining the possible hazards and possible routes of exposure in conjunction (i.e., Risk = Hazard x Exposure). In events where exposure may be possible, but no hazard is identified, risk is concluded to be negligible.

EPA concluded that the potential of exposure of any nontarget organisms, which necessarily includes endangered and threatened species, to OX5034 *Ae. aegypti* male mosquitoes is limited.

EPA concluded that the consumption of OX5034 *Ae. aegypti* male mosquitoes by nontarget organisms is not expected to pose a hazard to any nontarget organisms, which necessarily includes endangered or threatened species.

EPA concluded that the possible reduction of the *Ae. aegypti* populations in the EUP locations is not expected to pose a hazard to any nontarget organisms, which necessarily includes endangered or threatened species.

Therefore, although exposure may be possible (but is expected to be limited), and because no hazard was identified (i.e., no hazard from oral consumption or from the reduction of the local *Ae. aegypti* population), EPA concluded that no adverse effects are anticipated for nontarget organisms as a result of the experimental permit to release OX5034 *Ae. aegypti* male mosquitoes. Therefore, since adverse effects are not anticipated to any nontarget organism, a "No Effect" determination is also made for direct and indirect effects to federally listed endangered and threatened species, and for their designated critical habitats.